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(54) Abstract Title: **Screening method for a binding molecule**

(57) A method for identifying a binding molecule that binds to a target site of a target molecule comprising providing a target molecule comprising a target site to which is bound a tag moiety to form a tag complex, contacting this target complex with a plurality of candidate binding molecules and selecting a binding molecule that binds simultaneously to the tag and the target molecule. The candidate binding molecule is then modified in the region that interacts with the tag to form a plurality of modified binding molecules, and these modified binding molecules are then contacted with the target molecule in the absence of the tag moiety. The modified candidate binding molecules that bing the target in the absence of the tag are then selected. The binding molecule is preferably an antibody, and the target molecule is preferable and antigen, an antibody, a hapten or a tag.

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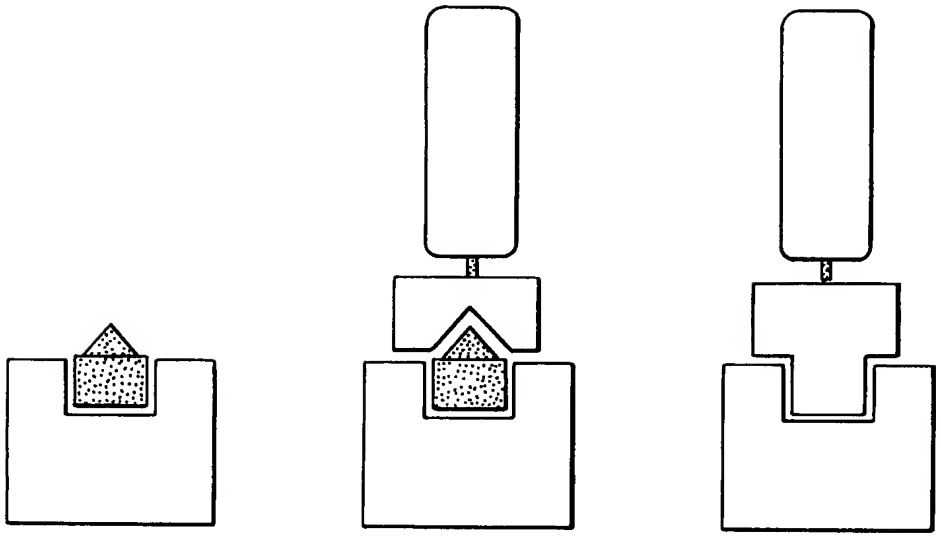


Fig. 1

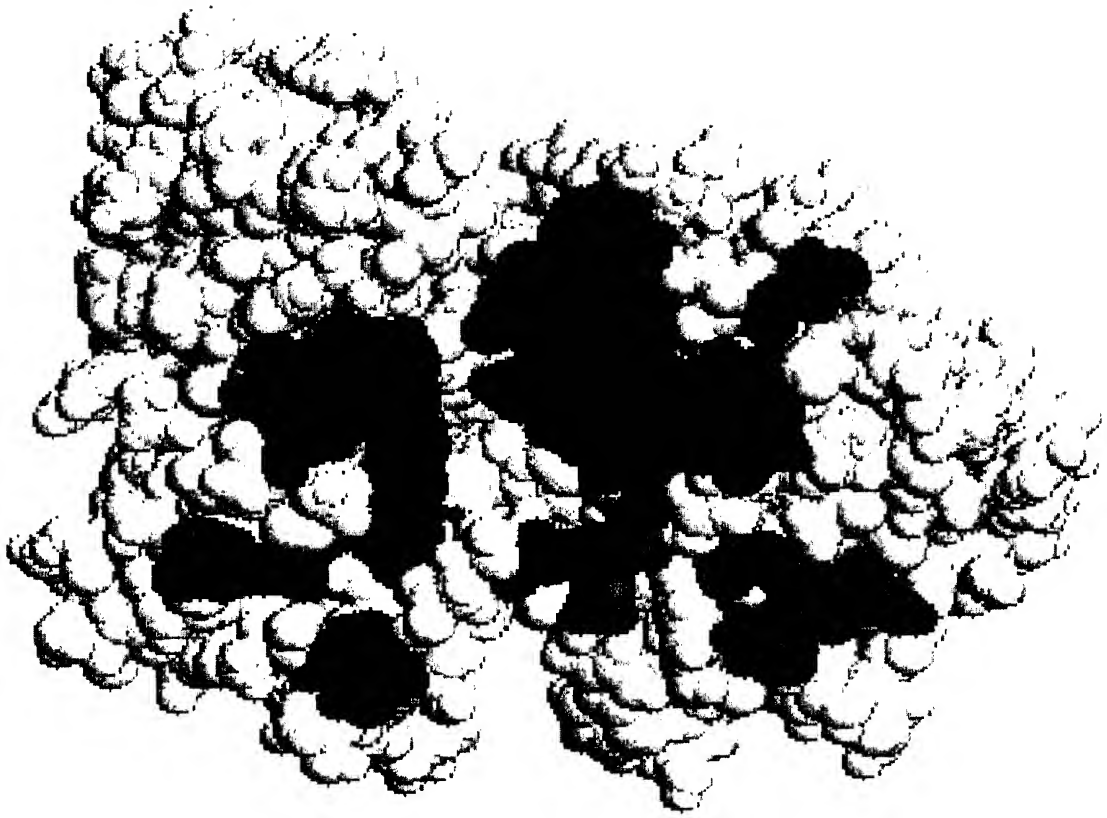


Fig. 2A

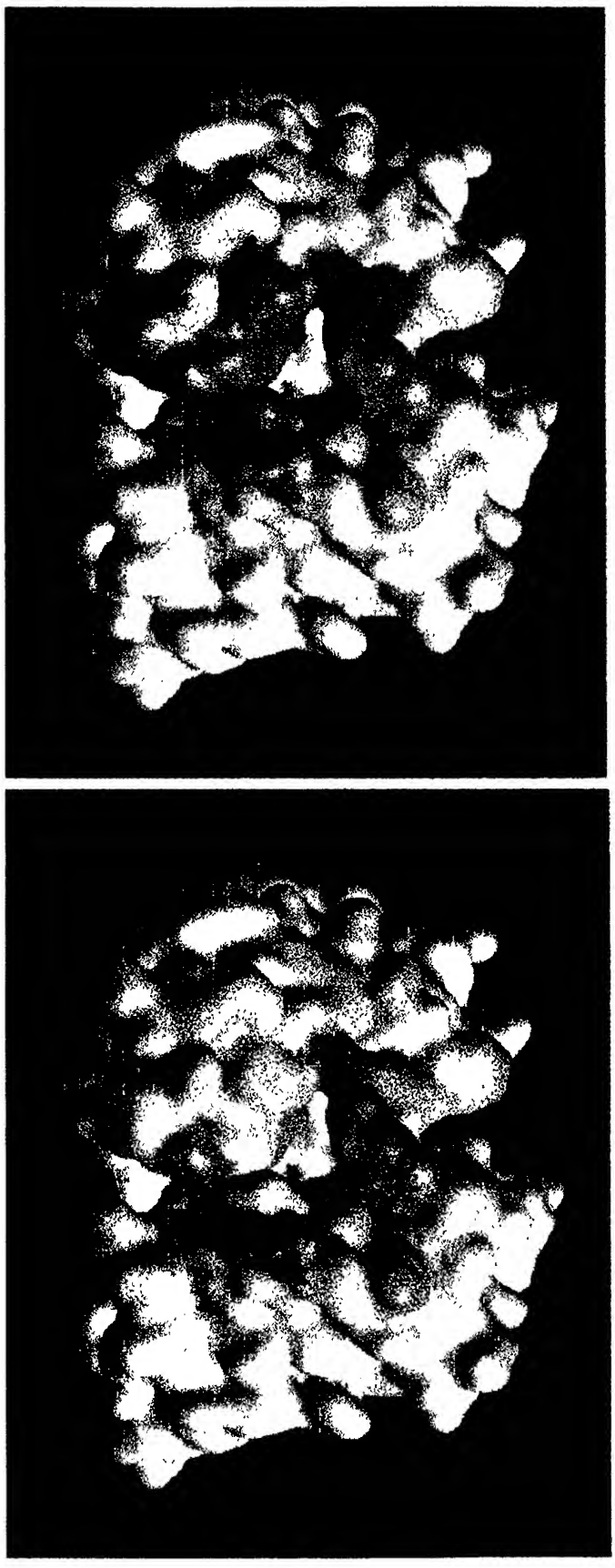
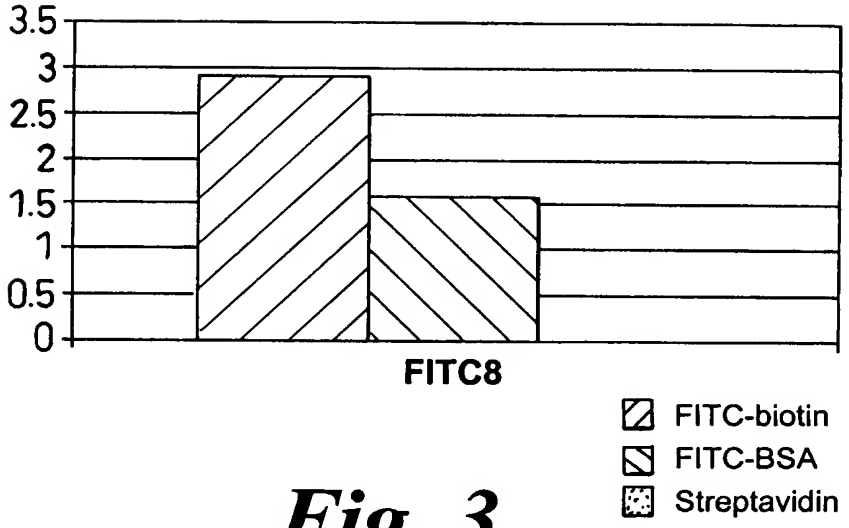


Fig. 2B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3
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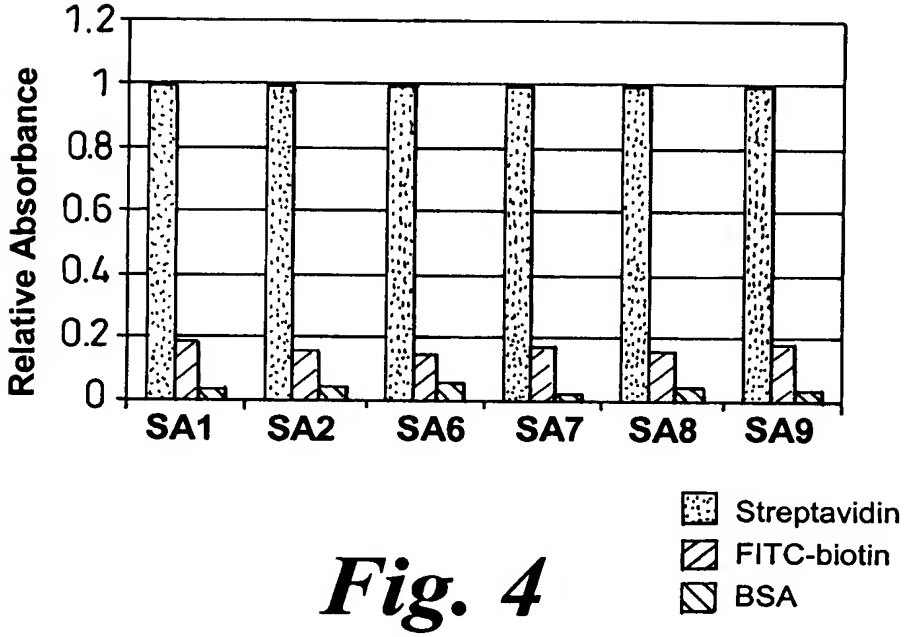
2
1.5
1
0.5
0
FITC8
FITC-biotin
FITC-BSA
Streptavidin

Fig. 3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1
0.8
0.6
0.4
0.2
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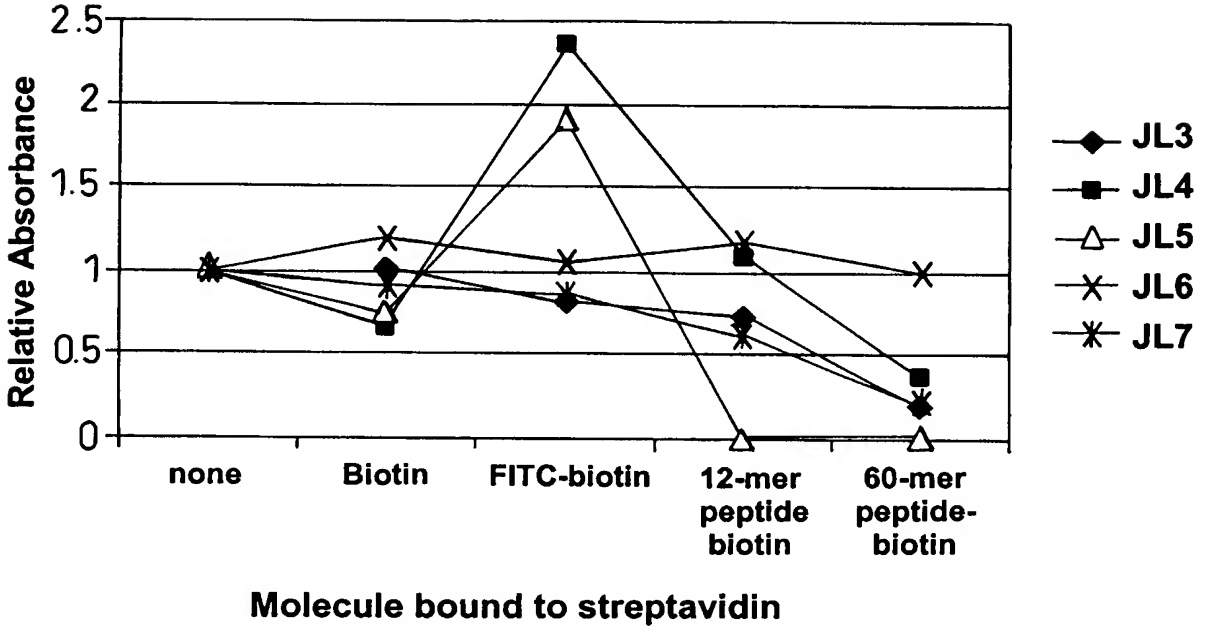
SA2
SA6
SA7
SA8
SA9
Streptavidin
FITC-biotin
BSA

Fig. 4


2.5
2
1.5
1
0.5
0
Relative Absorbance
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Biotin
FITC-biotin
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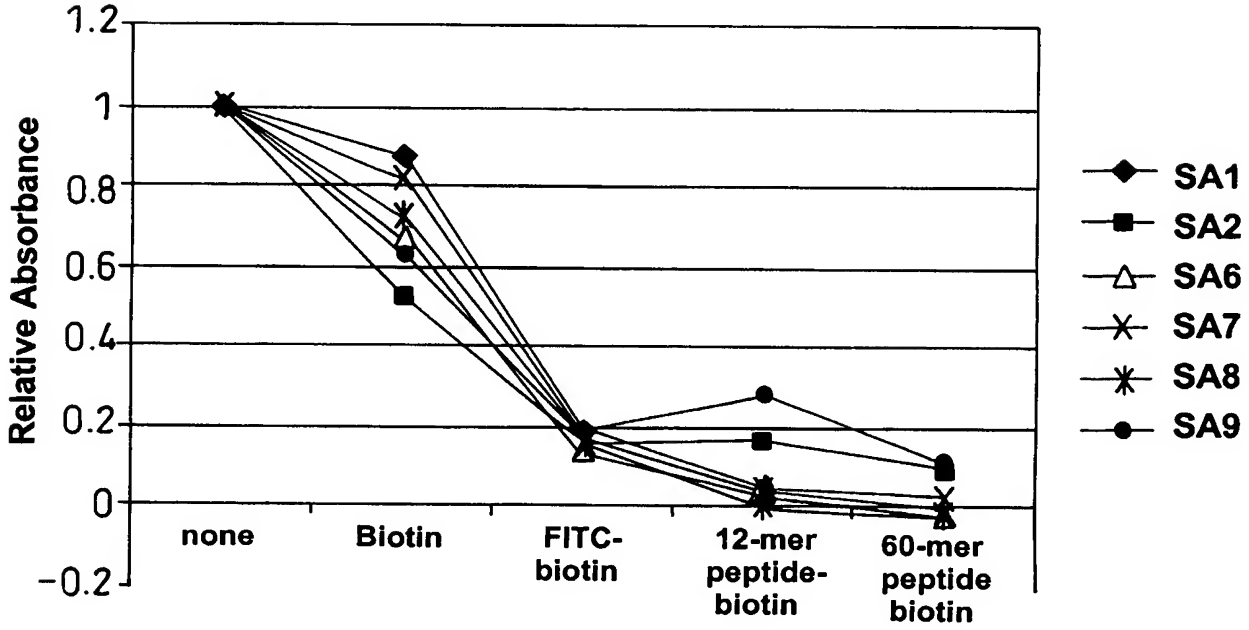
60-mer peptide-biotin
Molecule bound to streptavidin
JL3
JL4
JL5
JL6
JL7

Fig. 5A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0.4
0.2
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Relative Absorbance
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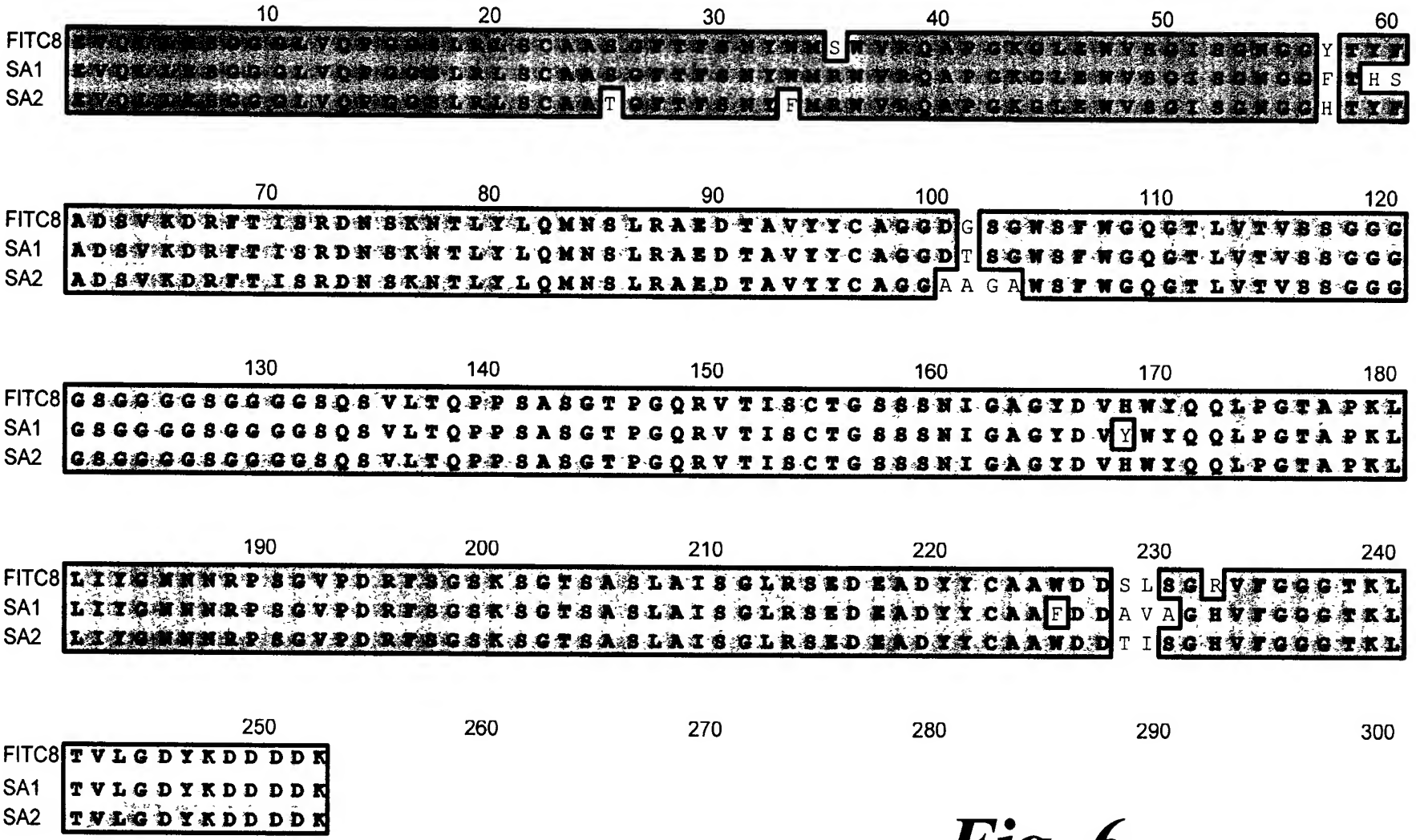
60-mer peptide biotin
Molecule bound to streptavidin
SA1
SA2
SA6
SA7
SA8
SA9

Fig. 5B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SA1
SA2
70 80 90 100 110 120
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SA1 ADSVKDRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGGDTSGWSFWGQGTLVTVSSGGG
SA2 ADSVKDRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGGAAGAWSFWGQGTLVTVSSGGG
130 140 150 160 170 180
FITC8 GSGGGGSGGGGSQSVLTQPPSASGTPGQRVTISCTGSSSNIGAGYDVHWYQQLPGTAPKL
SA1 GSGGGGSGGGGSQSVLTQPPSASGTPGQRVTISCTGSSSNIGAGYDVYWYQQLPGTAPKL
SA2 GSGGGGSGGGGSQSVLTQPPSASGTPGQRVTISCTGSSSNIGAGYDVHWYQQLPGTAPKL
190 200 210 220 230 240
FITC8 LIYGNNNRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLSGRVFGGGTKL
SA1 LIYGNNNRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAFDDAVAGHVFGGGTKL
SA2 LIYGNNNRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDTISGHVFGGGTKL
250 260 270 280 290 300
FITC8 TVLGDYKDDDDK
SA1 TVLGDYKDDDDK
SA2 TVLGDYKDDDDK

Fig. 6

SCREENING METHODS

Field of the invention

The present invention relates to a method for identifying/selecting binding molecules that bind to a target site on a target molecule of interest.

Background to the invention

Life processes depend on functional interactions of a variety of molecules like proteins, nucleic acids, lipids, carbohydrates and a variety of other molecular entities. Surfaces of such molecules interact by physiochemical forces not only involving covalent bonds but also through electrostatic interactions, hydrogen bonds, hydrophobic interactions, and van der Waals interactions. Such interactions regulate the activities of cellular components and allow cells to receive signals from its environment and to act in relation to these signals. Receptors respond to binding by their ligands, resulting in transmission of signals that change the activity of other molecules that establish or break other molecular interactions eventually affecting processes within the cell.

Other molecules act outside of cells initiating interactions with specific targets resulting in a variety of effects like removal of foreign matter. In all these cases more or less complementary surfaces are kept in close proximity by a variety of forces like those stated above. The close proximity ensures that the interactions provide a favourable energy contribution to the formation of the complex.

Identifying molecules that bind to target molecules of interest is of key importance in a whole range of experimental and therapeutic procedures. A variety of methodologies are available to generate and screen for molecules that bind to a target molecule. However, a target molecule, especially if large, will have a number of surfaces where other molecule can bind. For example, upon immunisation of an animal with a

target molecule, or following selection of an antibody library by phage display, ribosomal display or an equivalent methodology, a range of antibodies with different specificities are generally obtained. These will be directed to various surfaces of the molecule in question. It is difficult to raise specifically, an antibody that binds to a particular target site on the target molecule. One possible approach might be to use antibodies that have been mapped as binding to other parts of the target molecule, to block other sites so that antibodies that bind to the site of interest can be selected (Ditzel *et al.*, 1995, J. Immunol. 154, 893-906). However, not only does one need access to specific antibodies covering other epitopes on the target molecule, but even then it is not necessarily possible to direct the selected molecules towards the target site of interest. Thus, at present, there are no satisfactory techniques available for generating molecules that bind to a selected target site on a molecule or molecular complex of interest.

Summary of the invention

Interacting molecules have complementary surfaces that allow a more or less stable interaction to occur. The general principles governing such interactions are also similar between different types of molecules. Antibodies form a group of variable interacting molecules for which there is extensive molecular interaction data. However, although the following discussion describes the interaction between antibodies and their antigens, the general principles apply to other types of interacting molecules as well.

A molecular binding site can be seen as being composed of different regions that *in vivo* have different roles in the development of a specific binding site. Antigen contacts are more common at amino acid residues located centrally in the antigen-binding site, while more peripheral residues are only contacted by large antigens. When selecting antibodies (*in vivo* or *in vitro*) specific for a low molecular weight molecule (hapten), recognition by the antibody of the hapten is often accompanied by recognition of the

linker connecting the hapten and, more importantly, of the protein (carrier) to which the hapten is coupled. This is based on the fact that in many cases a small antigen will not occupy the entire antigen-binding surface of the antibody (about 1000 $Å^2$). The hapten will insert itself into a cavity formed by the antigen-binding site and only interact with the central parts of the binding site. This leaves room for contacts to be made between the carrier and the more peripherally located residues of the binding site. Similarly, a short peptide that shows a detectable binding to an antibody may still only represent a part of the total binding site presented by the intact protein from which the peptide is derived.

A binder selected for binding the intact molecule thus carries a surface complementary not only to the peptide that represents a "linear epitope" but often also to other parts of the intact target. In conclusion, a small molecule occupies only a part of a potential binding site (on an antibody or more generally on a binding protein) leaving room for additional contacts to be established.

The present invention is based upon a realisation that this fact can be exploited for the development of specific binders targeting a surface of a molecule in the vicinity of an artificially introduced small molecule or sequence and that such binders can be diversified so as to recognise the native, non-modified molecule. The present invention thus provides a means for generating and selected binding molecules that bind to, or in the immediate vicinity of, a particular target site on a molecule or molecular complex of interest.

The approach described herein makes use of the ability of a molecule to use only part of its potential binding site for the interaction with a small molecule while leaving the rest of the surface free and able to make contacts with the larger molecule. In this approach the small molecule is used as a tag to direct the selection of binders to a pre-defined site of a target molecule, which is the ultimate target for which a binder is to be

developed. A binder specific for the small molecule or for the small molecule in complex with the carrier is used as starting point in the subsequent evolution of specific binders recognising the carrier in the absence of the small molecule.

Accordingly, the present invention provides a method for identifying a binding molecule that binds to a target site of a target molecule which method comprises:

(a) providing a target molecule comprising a target site to which is bound a tag moiety to form a target complex;

(b) contacting the target complex with a plurality of candidate binding molecules;

(c) selecting a candidate binding molecule that binds simultaneously to the tag moiety and the target molecule;

(d) modifying at least a part of a region of the selected candidate binding molecule that interacts with the tag moiety to form a plurality of modified binding molecules;

(e) contacting the plurality of modified binding molecules with the target molecule in the absence of the tag moiety; and

(f) selecting a modified candidate binding molecule which binds to the target molecule in the absence of the tag moiety.

Optionally, the affinity and/or specificity of a modified candidate binding molecule selected in step (f) for the target site of the target molecule is increased by molecular evolution.

In one embodiment the tag moiety is covalently bound to the target molecule. In another embodiment, the tag moiety is non-covalently bound to the target molecule.

The tag moiety may, for example, comprise a ligand for the target site of the target molecule.

The tag moiety may further comprise an adaptor moiety, which serves to bind in step (b), one or more binding molecules, but is not able to bind to the target site unless linked to another molecule. Examples of adaptor molecules include haptens and epitopes.

Preferably, in step (d), regions of the candidate binding molecule that interact with the target molecule but not the tag moiety are not modified.

The modification of the selected candidate binding molecule in step (d) is typically performed using random and/or site-directed mutagenesis.

In one embodiment, the plurality of candidate binding molecules used in step (b) are obtained by modifying a selected binding molecule which binds specifically to the tag moiety in the absence of the target molecule. Preferably, the plurality of candidate binding molecules are obtained by modifying one or more regions of the selected binding molecule other than regions that interact with the tag molecule.

Typically, modification of the selected binding molecule to generate the plurality of candidate binding molecules used in step (b) is performed using random and/or site-directed mutagenesis.

In a preferred embodiment, the plurality of candidate binding molecules are polypeptides, for example polypeptides comprising an immunoglobulin complementarity determining region (CDR), such as antibodies or fragments thereof.

In a second, related aspect, the present invention also provides a method for selecting binding molecules that bind specifically to a target site on a target molecule when that site is occupied by a ligand for that target molecule. In this method, the ligand is no longer part of the tag moiety but is instead, in effect, part of the target molecule. The adaptor molecule constitutes the tag moiety. Thus, the ligand is present throughout the screening process whereas the adaptor molecule is omitted in the second screening stage as in the first aspect of the invention.

Accordingly, the present invention further provides a method for identifying a binding molecule that binds specifically to a target site of a target molecule, when said target site is occupied by a ligand for said target molecule, which method comprises:

(a) providing a target molecule comprising a target site to which is bound a ligand to form a target complex, the ligand being bound to an adaptor moiety;

(b) providing a selected binding molecule which binds specifically to the adaptor moiety;

(c) modifying one or more regions of the selected binding molecule other than regions that interact with the adaptor moiety, to obtain a first plurality of candidate binding molecules

(d) contacting the target complex with the first plurality of candidate binding molecules under conditions that are sub-optimal for binding of the unmodified selected binding molecule to the adaptor moiety;

(e) selecting a candidate binding molecule that binds simultaneously to the moiety molecule and the target molecule;

(f) modifying at least a part of a region of the selected candidate binding molecule that interacts with the adaptor moiety to obtain a second plurality of candidate binding molecules;

(g) contacting the second plurality of candidate binding molecules with the target molecule bound to the ligand in the absence of the adaptor moiety; and

(h) selecting a candidate binding molecule which binds to the target molecule.

A particular use for this method is to evolve an antibody that can recognise the paratope of another antibody in complex with an antigen, particularly a low molecular weight antigen. Thus, in a particular embodiment, the present invention provides a method for selecting an

antibody that binds to a target antigen only when the antigen is present in an antibody-antigen complex, which method comprises:

(a) providing a first antibody which binds specifically to a hapten;

(b) providing a second antibody which binds specifically to the target antigen;

(c) modifying one or more regions of the paratope of said first antibody which do not contact the hapten when the unmodified antibody is bound to the hapten, to obtain a first plurality of modified antibodies;

(d) contacting the first plurality of modified antibodies with an antibody-antigen-hapten complex comprising the second antibody, the hapten and the target antigen under conditions that are sub-optimal for binding of the unmodified first antibody to the hapten;

(e) selecting a candidate antibody that binds simultaneously to the hapten and the second antibody;

(f) modifying at least a part of a region of the selected candidate antibody that interacts with the hapten to obtain a second plurality of candidate antibodies;

(g) contacting the second plurality of candidate antibodies with the second antibody bound to the target antigen in the absence of the hapten; and

(h) selecting a candidate binding molecule which binds to the second antibody.

The present invention also provides a binding molecule, such as an antibody, identified by any of the methods of the invention.

Throughout this specification, preferred aspects and embodiments apply, as appropriate, separately from, or in combination with, other aspects and embodiments, mutatis mutandis, whether or not explicitly stated as such.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques which are used for molecular, genetic, biochemical and chemical methods are described in, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference).

The screening method of the present invention is essentially a two-stage process. In the first stage, a molecular tag is used, which is bound at a target site on a target molecule to direct the selection of binding molecules that can bind to the target site. The molecular tag is selected to be of a suitable size so that it does not completely fill the potential binding surface of the binding molecules that are being screened. This is to allow binding molecules to form contacts with both the tag and regions of the target molecule at, or proximal to, the target site at which the tag is bound (see Figure 1B).

By way of example, the epitope of an antigen interacts with the antigen-binding surface, the paratope, of an antibody. Epitope-binding sites are formed largely by six loops, complementarity determining regions (CDR), held in place by a number of beta-sheets. As the sequence and length of the loops vary between antibodies they can form different surfaces with respect to shape and the location of a variety of residues being able to mediate different types of interactions with other molecules. Consequently, small molecules, haptens, tend to bind into cavities formed by the paratope, elongated molecules bind to groves formed in the paratope while large

proteins surfaces bind to a flat paratope surface. Other protein-based binders behave similarly. For example, crystalline carbohydrates forming flat surfaces tend to interact favourably with flat carbohydrate-binding protein surfaces while non-crystalline polysaccharides, like xylane, bind to cleft-like carbohydrate-binding protein surfaces. Thus many characteristics of binding surfaces of diverse proteins are similar in nature.

In the case of binding molecules based on immunoglobulin CDRs, the binding surface of such molecules, the paratope, has an area of about 1000 $Å^2$. Thus, the tag should occupy less than 1000 $Å^2$ when bound to the CDR, preferably less than 800, 700 or 600 $Å^2$. In more general terms, it is preferred that the tag should occupy less than 80, 70 or 60% of the binding surface of the binding molecules which are being screened, so as to allow contacts to be made between the binding molecules and the target molecule when the tag is bound to the binding molecule in the first stage of the screening process.

The molecular tag comprises a moiety which is bound to the target site of the target molecule. In one embodiment, the tag comprises a ligand which binds at the target site. For example, where the target molecule is a receptor, the tag comprises a ligand for that receptor; where the target molecule is an enzyme, the tag comprises a substrate for the enzyme, which may be an analogue of the natural substrate to prevent the enzyme from modifying the substrate during the screening process; where the target molecule is an antibody, the ligand is an antigen. In general, these tags are bound non-covalently to the target molecule.

Alternatively, or in addition, the tag moiety may be covalently linked to the target molecule. For example, polypeptide targets may be engineered to include a peptide tag at the target site.

The tag may also comprise an adaptor moiety. The function of the adaptor is to serve as a target for the binding molecules in the first stage of the screening process. This is particularly advantageous where binding

molecules are available that are specific for the adaptor but binding molecules are not available that are specific for the part of the tag that binds to the target site of the target molecule. An example of a suitable adaptor moiety is a hapten or a small antigen where the binding molecules comprise immunoglobulin CDRs or the like.

Target molecules

Target molecules include peptides, polypeptides, nucleic acids and carbohydrates.

Peptides and polypeptides include receptors, enzymes and polypeptides which comprise an immunoglobulin complementarity determining region (CDR), e.g. antibodies, antigen receptors, and fragments and derivatives thereof.

In the screening methods of the invention, target molecules may conveniently be immobilised to a solid phase, such as a chromatographic resin e.g. agarose, or a substantially planar surface such as a chip. This may be achieved by any suitable means, such as the use of an affinity tag or standard covalent coupling methodologies.

Candidate binding molecules

Candidate binding molecules include peptides, polypeptides, nucleic acids, and carbohydrates. Preferred peptides and polypeptides comprise an immunoglobulin complementarity determining region (CDR), e.g. antibodies, antigen receptors, and fragments and derivatives thereof.

Pluralities of candidate binding molecules are typically libraries of diversified molecules, such as libraries of partially or fully randomised molecules. It is preferred to randomise/diversify only those regions of the molecules that form part of the binding surface of the molecules and will therefore potentially make contact with the target molecule/tag moiety. Thus, for example, in the case of antibodies, those regions of the antibody

that constitute the paratope are typically varied. Techniques for generating libraries of different molecules are well known in the art and will be described in more detail below.

In one embodiment, the plurality of candidate binding molecules is derived from a selected binding molecule that binds specifically to the tag moiety. The binding molecule is then subjected to diversification to generate a plurality of molecules that differ from one another. For example, one or more regions of the binding molecule can be varied on a partially or fully random basis. The diversification process should result in a plurality of molecules that have different binding properties which can then be screened to identify a suitable binding molecule that binds to the tag moiety and a region of the target molecule at or proximal to the target site.

This is typically achieved by varying regions of the binding molecule that form the binding surface, although variations elsewhere can also cause changes in binding properties due to allosteric effects.

Since, as discussed above, the tag moiety is of a size such that it does not occupy the entire binding surface of the binding molecules being screened, the binding surface of the selected binding molecule will comprise residues that contact the tag moiety and residues that do not. When diversifying the sequence of the selected binding molecule. It is therefore preferable to vary at least those residues of the binding surface selected binding molecule that are capable of making contacts with the target molecule, i.e. those residues that do not contact the tag moiety. Further, it is preferred not to vary all or most of those residues that do interact with the tag moiety.

Candidate binding molecules may be produced linked to another molecule or molecular complex that facilitates their selection, purification and/or identification during the screening process. For example, polypeptide binding molecules are preferably physically linked to a nucleic acid that encodes the polypeptide. The polypeptide is therefore presented in

a format whereby it is linked to its corresponding nucleic acid. Examples of techniques that use this approach include bacterial display, phage display and ribosome display technology or similar techniques. Further information on the principles and practice of phage display is provided in *Phage display of peptides and proteins: a laboratory manual* Ed Kay, Winter and McCafferty (1996), the disclosure of which is incorporated herein by reference. Binding molecules can also be produced linked to affinity tags, e.g. as fusion proteins in the case of polypeptide binding molecules.

Candidate binding molecules may also be immobilised to a solid phase. For example, libraries of candidate binding molecules can be immobilised to generate an array. In one embodiment, the array is spatially addressable such that the identity of a candidate binding molecule at a given position in the array is known.

Diversification of binding molecules to generate libraries of molecules

Binding molecules can be subjected to a range of techniques, well known in the art, to modify, diversify and evolve molecules. Polynucleotides and polypeptides encoded by polynucleotides can conveniently be modified by random and/or site-directed mutagenesis. Random mutagenesis can be performed using, for example, chemical mutagens or mutator bacterial strains. A number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Other methods including shuffling techniques such as chain shuffling, CDR shuffling or DNA shuffling (e.g Stemmer, 1994a, Nature 370: 389-9; Stemmer, 1994b, PNAS 91: 10747-51). Diversity can also be introduced using synthetic residues during solid phase synthesis.

Other polymeric binding molecules, such as carbohydrates, can be diversified during synthesis, by for example adding mixtures of subunits at some or all chain extension steps.

Selection of binding molecules that bind simultaneously to the target molecule and the tag moiety.

In the first screening step, the plurality of candidate binding molecules are contacted with a complex of the target molecule and the tag moiety, bound to the target site of the target molecule. The screening process selects for those candidate binding molecules that bind both to the target complex and to the tag moiety since such molecules are likely to be making contacts with the target complex and to the tag moiety simultaneously.

In general, selection is achieved by identifying those candidate binding molecules that bind to the target complex. However, some of these candidate binding molecules may bind to the tag moiety alone or to the target molecule alone. These candidate binding molecules can be eliminated by one or more negative selection steps in the presence of the tag moiety alone or to the target molecule alone. The various selection steps, positive and negative, can be performed in any order.

Where the candidate binding molecules are derived from a selected binding molecule specific for the tag moiety, selection can advantageously be achieved by incubating the candidate binding molecules and the target complex under conditions which are sub-optimal for the binding of the original unmodified binding molecule to the tag moiety since additional complementary binding capacity will be required to form a stable complex. Again, if necessary, selected molecules can be subject to negative selection with the tag moiety and/or the target molecule alone to remove candidate binding molecules that do not require binding to both the target molecule and the tag moiety simultaneously.

In the second stage of the selection process, a candidate binding molecule identified in the first stage is modified to introduce further binding diversity, in particular in the region of the binding surface of the molecule that interacts with the molecular tag but not in the region that interacts with the target molecule. The tag is then omitted from the screening process. The residues in the binding surface of the candidate binding molecule selected in the first stage, act as a molecular anchor to direct binding to the target site stage. However, the selection procedure takes place under conditions where further suitable contacts are required to form a stable complex. Consequently, the screening process serves to identify modified/diversified candidate binding molecules that possess additional complementarity to the target site in the region that in the first stage bound specifically to the tag moiety (see Figure 1C).

Diversification of the candidate binding molecule can be achieved in a similar manner to that described in the first stage. However, as discussed above, modifications/ randomisation should be directed to those residues that make contact with the tag moiety and avoid if possible, all or most of those residues that contact the target molecule.

Selection of binding molecules that bind to the target molecule in the absence of the tag moiety.

In the second screening step, the plurality of modified candidate binding molecules are contacted with only the target molecule i.e. in the absence of the tag moiety. This step makes use of the target-specific parts of the binding molecules selected in the first stage whilst adapting the other regions of the binding surface to the regions of the target site now exposed following removal of the tag moiety. Since the candidate binding molecules possess binding complementarity with residues in and around the target site on the target molecule, candidate binding molecules selected as binding to

the target molecule will typically do so at, or proximal to, target site of interest (see figure 1C).

Binding molecules selected by the methods of the invention may be subjected to further rounds of diversification/selection. Alternatively, or in addition, molecular evolution methodologies may be used to further improve the affinity/specificity of the selected binding molecules. For example two or more selected binding molecules may be subjected to DNA shuffling.

It should be appreciated that reference herein to binding molecules that bind to the target site of the target molecule also includes binding molecules that bind close to the target site. However, it is preferred that binding molecules identified by the methods of the invention bind to the target molecule such that at least a part of the binding molecule prevents ligands binding to the target site. This can, for example, be determined by incubating a selected binding molecule with the target molecule, contacting the binding molecule/target molecule complex with a ligand that can bind to the target site of the target molecule in the absence of the binding molecule and determining whether the ligand can bind. A lack of ligand binding indicates that the binding molecule is blocking the target site.

In general, it is preferred that the binding molecule binds within 500 Å of the target site, more preferably within 200 or 100 Å of the target site.

Selection of binding molecules that recognise specifically ligands when bound to their target molecule

This method differs from the method described above in that although a ligand for the target molecule is used in the screening process, the ligand is no longer part of the tag moiety but is instead, in effect, part of the target molecule. The adaptor molecule constitutes the tag moiety.

Thus, although the adaptor molecule is omitted in the second screening stage, the ligand is present throughout the screening process.

In the first stage, a plurality of diversified candidate binding molecules is derived from a selected binding molecule that is specific for an adaptor molecule. The plurality of diversified candidate binding molecules is screened against a complex of the target molecule, a ligand that binds to the target molecule and the adaptor molecule, which is bound to the ligand.

In the second stage, the adaptor molecule is omitted. It is desirable during the second screening stage to carry out a negative selection in the presence of the target molecule alone, i.e. in the absence of the ligand, to eliminate selected candidate molecules that do not require the presence of the ligand bound to the target.

In a preferred embodiment, the target molecule is an antibody, with the ligand being an low molecular weight antigen to which the antibody binds. The adaptor molecule is a hapten for which a specific antibody is available (or is generated). The end result in this case is an antibody that only binds to the antibody-antigen complex and not to the antibody or antigen alone. The hapten serves as the tag moiety. This embodiment is further described in Example 2.

The invention will be further described by way of example, with reference to the following, non-limiting, specific embodiments

1. Diversification of a hapten-specific binder so as to make it into a target site-specific binder.

a. Make use of a known ligand for the active site of the target molecule. This may e.g. include a low molecular weight hormone binding a receptor, or an agonist or a competitive antagonist that binds the active site of the target. This molecule is modified so as to carry a hapten to which a binder such as a scFv is available. Based on this clone, create a library containing diversity in the paratope of the binder. If possible but not necessarily, one

should attempt to restrict diversity to those parts of a potential paratope that are believed not to be involved in making contact with the hapten. Create a library of displayed molecules and select those variants from the library that specifically interact with the target/ligand-hapten complex under conditions were the original binder does not bind well to the ligand-hapten itself. Instead complementary binding capacity provided by the target allows for the complex to be formed and maintained.

b. Create a new library based on the clone(s) selected above incorporating diversity in the antigen-binding site. This can be done by any available methodology such as, but not limited to, random mutagenesis, introduction of synthetic or natural diversity, chain shuffling, CDR shuffling or DNA shuffling and should if possible mainly target those parts of a potential paratope that are believed to be involved in making contact with the hapten. The hereby-created diversity is now selected on the target in the absence of the ligand-hapten complex (or in the presence of the ligand alone). This approach makes use of the target-specific part of the originally selected clone(s) and adapts the paratope to the now missing ligand-hapten complex by the introduction of diversity in the binding site. Selected clones may be further improved by molecular evolution by using any of the available methods known to skilled researchers in the field.

2. Use of ligand-hapten to direct a selection of binders to the active site of a target.

a. Make use of a known ligand for the active site of the target. This may e.g. include a low molecular weight hormone which binds to a receptor, or an agonist or a competitive antagonist that binds to the active site of the target. This molecule is modified so as to carry a hapten. Allow the ligand-hapten to bind to the target and select a binder specifically recognising the ligand-hapten complex bound to the target from any diverse

antibody library. Clones recognising this structure may be enriched by elution of specific clones recognising the active site by elution with the hapten or the hapten-ligand conjugate. Alternatively, the selection procedure may involve an intermediate selection step(s) on hapten coupled to another carrier. These procedures will eliminate clones that preferentially recognise other parts of the target.

b. Create a new library based on the clone(s) selected above incorporating diversity in the antigen-binding site. Again, this can be done by any available methodology such as, but not limited to, random mutagenesis, introduction of synthetic or natural diversity, chain shuffling, CDR shuffling or DNA shuffling and should if possible mainly target those parts of a potential paratope that are believed to be involved in making contact with the hapten. The hereby-created diversity is now selected on the target in the absence of the ligand-hapten complex (or in the presence of the ligand alone). This approach makes use of the target-specific part of the originally selected clone(s) and adapts the paratope to the now missing ligand-hapten complex by the introduction of diversity in the binding site. Selected clones may be further improved by molecular evolution by using any of the available methods known to skilled researchers in the field.

3. Diversification of a ligand-specific binder so as to make it into a target-specific binder.

a. Make use of a known ligand containing at least two different structures; one a target-binding structure and the other an epitope to which a binder such a scFv is available. The method requires that the size of the ligand is large enough to allow simultaneously binding to the target as well as to the scFv. However, there is also an upper size limit of the ligand. Too large a ligand will occupy the whole antigen-binding surface thus preventing contacts to be made between the paratope and the target. Based

on the ligand specific svFv, create a library containing diversity in the paratope of the binder. If possible but not necessarily, one should attempt to restrict diversity to those parts of a potential paratope that are believed not to be involved in making contact with the ligand. Create a library of displayed molecules and select those variants from the library that specifically interact with the target -ligand complex under conditions were the original binder does not bind well to the ligand itself. Instead complementary binding capacity provided by the target allows for the complex to be formed and maintained.

b. Create a new library based on the clone(s) selected above incorporating diversity in the antigen-binding site. This can be done by any available methodology such as, but not limited to, random mutagenesis, introduction of synthetic or natural diversity, chain shuffling, CDR shuffling or DNA shuffling and should if possible mainly target those parts of a potential paratope that are believed to be involved in making contact with the ligand. The hereby-created diversity is now selected on the target in the absence of the ligand. This approach makes use of the target -specific part of the originally selected clone(s) and adapts the paratope to the missing ligand by the introduction of diversity in the binding site. Selected clones may be further improved by molecular evolution by using any of the available methods known to skilled researchers in the field.

4. **Use of molecular tagging to direct a selection of binders to the active site of a target.**

a. The active site or the vicinity of the active site of the target is modified so as to carry a tag, a peptide/protein sequence, to which a binder such as a scFv is available. Based on this scFv, create a library containing diversity in the paratope of the binder. If possible but not necessarily, one should attempt to restrict diversity to those parts of a potential paratope that

are believed not to be involved in making contact with the tag. Create a library of displayed molecules and select those variants from the library that specifically interact with the target-tag complex under conditions were the original binder does not bind well to the tag itself.

b. Create a new library based on the clone(s) selected above incorporating diversity in the antigen-binding site. This can be done by any available methodology such as, but not limited to, random mutagenesis, introduction of synthetic or natural diversity, chain shuffling, CDR shuffling or DNA shuffling and should if possible mainly target those parts of a potential paratope that are believed to be involved in making contact with the ligand. The hereby-created diversity is now selected on the target in the absence of the tag. This approach make use of the target-specific part of the originally selected clone(s) and adapt the paratope to the missing tag by the introduction of diversity in the binding site. Selected clones may be further improved by molecular evolution by using any of the available methods known to skilled researchers in the field.

The present invention will now be described in more detail with reference to the following examples, which are illustrative only and non-limiting.

Figure 1. Principle of evolution of target active site-specific binders using ligand-hapten complexes as a driving force. A target /ligand-hapten complex is formed (A). A specific binder is selected from a molecular library (of e.g. antibody fragments) on the target /ligand-hapten complex to obtain a variant that recognises the hapten as well as the target (B). A library of variants of the clone(s) selected in (B) is constructed and new variants specific for the target alone is selected (C).

Figure 2. Structure model of FITC8 highlighting residues carrying diversity.

A. Highlighted residues are those residues in CDRH3 that are diversified in the second-generation library (orange), residues diversified in the second-generation library (red) and other residues except for those found in CDRH3 that in n-CoDeR LIB2000 achieves a variability >10 (green).

B. Separate images of FITC8 with (left) only the level of diversity in LIB2000 shown in blue. CDHR3 residues between CAR and WGQG (residues 99-106 in the linear sequence of FITC8) are shown in orange. Other residues are color coded in blue to indicate the diversity of the library with an increased blue tone representing increased diversity (CMYK:[100%, 100%, 0%, 0%]= variability 50: CMYK:[2%, 2%, 0%, 0%]= variability 1). Residues that were diversified in the second-generation library are shown in light red (right). Variability was defined as described by Wu and Kabat, 1970, J. Exp. Med. 132, 211-250.

Figure 3. FITC8 scFv is able to recognise FITC irrespective of which carrier protein it is bound to (via biotin to streptavidin (blue) or directly to bovine serum albumin (red)). No detectable binding to streptavidin itself is seen though (yellow).

Figure 4. After selection of the library on streptavidin, randomly picked sequence variants were shown to recognise streptavidin (yellow). They did not efficiently bind to FITC-biotin bound to streptavidin (blue) or to a control protein (bovine serum albumin (green).

Figure 5. Reactivity of scFv selected for reactivity to the steptavidin binding site by the current approach (clone SA1, SA2, SA6, SA7, SA8, SA9) and for general binding to streptavidin without attempting to target the specificity to the binding site (clones JL3-7). Phages displaying scFv

were allowed to bind to streptavidin, or straptavidin to which biotin, FITC-biotin, a biotin-labelled 12-mer peptide or a biotin-labelled 60-mer peptide had been bound. Bound phages were detected using peroxidase-labelled mouse anti-M13 phage antbodies. ScFv fragments developed by the current approach were substantially inhibited in the ability to recognise strepatvidin even if the binding site was occupied by a low molecular weight molecule. In contrast, streptavidin-specific scFv selected by a conventional approach tolerated the presence of small molecules in the biotin-binding site and their recognition of strepatavidin was in general inhibited only if a large molecule (like biotin-MUC60) was bound to the biotin binding site.

Figure 6. Amino acid sequences of an initial scFv (FITC-8) selected for binding to FITC-biotin/streptavidin complexes and two scFv evolved from this clone into a streptavidin-specific scFv.

Example 1. Evolution of a streptavidin-binding antibody fragment binding in the immediate vicinity of the biotin-binding site.

This example relates to methodology of specific embodiment #2 described above, employing streptavidin as the target, biotin as the ligand and FITC as the hapten. ScFv had selected from the n-CoDeR library against FITC-biotin bound to streptavidin (Söderlind *et al.*, 2000, Nat. Biotechnol. 18, 852-856). These scFv variants represent the initial candidates that harbour a paratope that interacts not only with FITC but most likely also with streptavidin. A second library was created based on one such specific clone called FITC-8 introducing diversity mainly in the cavity believed to be involved in binding of the scFv to FITC. By viewing a model of FITC-8 one realises that the diversity in the secondary library mainly targets the central region of the paratope believed to interact with the hapten while the original library design carries diversity in other parts of the paratope as well (Figure 2), suggesting that diversity here could have been selected for

binding to streptavidin. However, the affinity for strepatavidin was too low to be detected (Figure 3).

This second library was selected on streptavidin coupled to Dynabeads® (Dynal AS, Oslo, Norway) and molecular variants specifically interacting with this protein were identified. The binding specificity of such scFv was determined demonstrating that they recognised streptavidin but had largely lost its binding to FITC (Figure 4). The specificity of these scFv was within/close to the region of the biotin-binding site of streptavidin, as demonstrated by the ability of small ligands bound via biotin to the biotin-binding site to block the interaction of the scFv with streptavidin. In contrast, scFv selected directly from n-CoDeR on streptavidin were in most cases refractory to the addition of a biotinylated ligand suggesting that they recognised other epitopes on streptavidin (Figure 5). The sequences of such scFv are shown in Figure 6.

Example 2. Evolution of an antibody recognising the paratope of another antibody in complex with an antigen.

An extension of this approach is the development of a specific antibody recognising an antibody-hapten complex, thus forming the basis for the development of a non-competitive analysis of haptens. Low molecular weight molecules are normally non-amenable to non-competitive assays as non-competitive assays require that a given antigen can bind more than one antibody at a time. Only under special circumstances is it possible to develop these kinds of highly sensitive assays (Hashida *et al.*, 1991, J. Biochem. 110, 486-492; Kobayashi *et al.*, 2003, J. Immunol. Methods. 274, 63-75). An interesting approach would be to develop antibodies against a hapten antibody complex itself. The development of such antibodies is however difficult to achieve as many other epitopes on an antibody will also allow for the selection of specific clones.

However, our approach allows the development of an antibody fragment (or any other molecules able to recognise an antigen-antibody complex) specific for an antibody in complex with a low-molecular weight antigen. An antigen-hapten complex, e.g. FITC-labelled steroid is bound to an antigen (steroid)-specific antibody. Specific binders are selected from a library of molecules made by diversification of a hapten-specific antibody. This library is created mainly by diversification of residues not believed to be involved in the binding of the hapten (e.g. FITC). Variants from the library that specifically interact with the antibody/antigen-hapten complex are selected under conditions where the original binder does not bind well to the hapten itself. Instead complementary binding capacity provided by the antibody-antigen complex allows for the complex to be formed and maintained.

A new library is created based on the clone(s) selected above incorporating diversity in the antigen-binding site, mainly targeting those parts of a potential paratope that are believed to be involved in making contact with the hapten. The hereby-created diversity is now selected on the antibody-antigen complex. Negative selection on antibody in the absence of antigen is carried out to eliminate clones not requiring antigen. Alternatively, selection is carried out on immobilised antibody-antigen complexes in the presence of free antibody to achieve the same result. This approach make use of the antibody-antigen (e.g. steroid)-specific part of the originally selected clone(s) and adapts the paratope to the now missing hapten by the introduction of diversity in the binding site.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as

claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method for identifying a binding molecule that binds to a target site of a target molecule which method comprises:

(a) providing a target molecule comprising a target site to which is bound a tag moiety to form a target complex;

(b) contacting the target complex with a plurality of candidate binding molecules;

(c) selecting a candidate binding molecule that binds simultaneously to the tag moiety and the target molecule;

(d) modifying at least a part of a region of the selected candidate binding molecule that interacts with the tag moiety to form a plurality of modified binding molecules;

(e) contacting the plurality of modified binding molecules with the target molecule in the absence of the tag moiety; and

(f) selecting a modified candidate binding molecule which binds to the target molecule in the absence of the tag moiety.

2. A method according to claim 1 wherein the tag moiety is covalently bound to the target molecule.

3. A method according to claim 1 wherein the tag moiety is non-covalently bound to the target molecule.

4. A method according to claim 3 wherein the tag moiety comprises a ligand for the target site of the target molecule.

5. A method according to claim 4 wherein the tag moiety further comprises an adaptor moiety.

6. A method according to any one of the preceding claims wherein in step (d), regions of the candidate binding molecule that interact with the target molecule but not the tag moiety are not modified.

7. A method according to any one of the preceding claims wherein in step (d) modification of the selected candidate binding molecule is performed using random and/or site-directed mutagenesis.

8. A method according to any one of the preceding claims wherein the plurality of candidate binding molecules are obtained by modifying a selected binding molecule which binds specifically to the tag moiety in the absence of the target molecule.

9. A method according to claim 8 wherein the plurality of candidate binding molecules are obtained by modifying one or more regions of the selected binding molecule other than regions that interact with the tag molecule.

10. A method according to claim 8 or claim 9 wherein modification of the selected binding molecule is performed using random and/or site-directed mutagenesis.

11. A method according to any one of claims 8 to 10 wherein in step (b) the target complex is contacted with the plurality of candidate binding molecules under conditions which are sub-optimal for binding of the selected binding molecule to the tag moiety.

12. A method according to any one of the preceding claims wherein the plurality of candidate binding molecules are polypeptides.

13. A method according to claim 12 wherein the polypeptides comprise an immunoglobulin complementarity determining region (CDR).

14. A method according to claim 13 wherein the adaptor moiety comprises a hapten or an epitope.

15. A method according to any one of the preceding claims wherein the target molecule is an enzyme, an antibody or a receptor.

16. A method according to any one of the preceding claims wherein the affinity and/or specificity of a modified candidate binding molecule selected in step (f) for the target site of the target molecule is increased by molecular evolution.

17. A binding molecule identified by the method of any one of the preceding claims.

18. A method for identifying a binding molecule that binds specifically to a target site of a target molecule, when said target site is occupied by a ligand for said target molecule, which method comprises:

(a) providing a target molecule comprising a target site to which is bound a ligand to form a target complex, the ligand being bound to an adaptor moiety;

(b) providing a selected binding molecule which binds specifically to the adaptor moiety;

(c) modifying one or more regions of the selected binding molecule other than regions that interact with the adaptor moiety, to obtain a first plurality of candidate binding molecules

(d) contacting the target complex with the first plurality of candidate binding molecules under conditions that are sub-optimal for binding of the unmodified selected binding molecule to the adaptor moiety;

(e) selecting a candidate binding molecule that binds simultaneously to the moiety molecule and the target molecule;

(f) modifying at least a part of a region of the selected candidate binding molecule that interacts with the adaptor moiety to obtain a second plurality of candidate binding molecules;

(g) contacting the second plurality of candidate binding molecules with the target molecule bound to the ligand in the absence of the adaptor moiety; and

(h) selecting a candidate binding molecule which binds to the target molecule.

19. A method for selecting an antibody that binds to a target antigen only when the antigen is present in an antibody-antigen complex, which method comprises:

(a) providing a first antibody which binds specifically to a hapten;

(b) providing a second antibody which binds specifically to the target antigen;

(c) modifying one or more regions of the paratope of said first antibody which do not contact the hapten when the unmodified antibody is bound to the hapten, to obtain a first plurality of modified antibodies;

(d) contacting the first plurality of modified antibodies with an antibody-antigen-hapten complex comprising the second antibody, the hapten and the target antigen under conditions that are sub-optimal for binding of the unmodified first antibody to the hapten;

(e) selecting a candidate antibody that binds simultaneously to the hapten and the second antibody;

(f) modifying at least a part of a region of the selected candidate antibody that interacts with the hapten to obtain a second plurality of candidate antibodies;

(g) contacting the second plurality of candidate antibodies with the second antibody bound to the target antigen in the absence of the hapten; and

(h) selecting a candidate binding molecule which binds to the second antibody.

Amendments to the claims have been filed as follows

1. A method for identifying a binding polypeptide that binds to a target site of a target polypeptide which method comprises:

(a) providing a target polypeptide comprising a target site to which is bound a tag moiety to form a target complex;

(b) contacting the target complex with a plurality of candidate binding polypeptides;

(c) selecting a candidate binding polypeptide that binds simultaneously to the tag moiety and the target polypeptide;

(d) modifying at least a part of a region of the selected candidate binding polypeptide that interacts with the tag moiety to form a plurality of modified binding polypeptides;

(e) contacting the plurality of modified binding polypeptides with the target polypeptide in the absence of the tag moiety; and

(f) selecting a modified candidate binding polypeptide which binds to the target polypeptide in the absence of the tag moiety.

2. A method according to claim 1 wherein the tag moiety is covalently bound to the target polypeptide.

3. A method according to claim 1 wherein the tag moiety is non-covalently bound to the target polypeptide.

4. A method according to claim 3 wherein the tag moiety comprises a ligand for the target site of the target polypeptide.

5. A method according to claim 4 wherein the tag moiety further comprises an adaptor moiety.

6. A method according to any one of the preceding claims wherein in step (d), regions of the candidate binding polypeptide that interact with the target polypeptide but not the tag moiety are not modified.

7. A method according to any one of the preceding claims wherein in step (d) modification of the selected candidate binding polypeptide is performed using random and/or site-directed mutagenesis.

8. A method according to any one of the preceding claims wherein the plurality of candidate binding polypeptides are obtained by modifying a selected binding polypeptide which binds specifically to the tag moiety in the absence of the target polypeptide.

9. A method according to claim 8 wherein the plurality of candidate binding polypeptides are obtained by modifying one or more regions of the selected binding polypeptide other than regions that interact with the tag moiety.

10. A method according to claim 8 or claim 9 wherein modification of the selected binding polypeptide is performed using random and/or site-directed mutagenesis.

11. A method according to any one of claims 8 to 10 wherein in step (b) the target complex is contacted with the plurality of candidate binding polypeptides under conditions which are sub-optimal for binding of the selected binding polypeptide to the tag moiety.

12. A method according to any one of the preceding claims wherein the plurality of candidate binding molecules comprise an immunoglobulin complementarity determining region (CDR).

13. A method according to claim 12 wherein the adaptor moiety comprises a hapten or an epitope.

14. A method according to any one of the preceding claims wherein the target polypeptide is an enzyme, an antibody or a receptor.

15. A method according to any one of the preceding claims wherein the affinity and/or specificity of a modified candidate binding polypeptide selected in step (f) for the target site of the target polypeptide is increased by molecular evolution.

16. A binding polypeptide identified by the method of any one of the preceding claims.

17. A method for identifying a binding polypeptide that binds specifically to a target site of a target polypeptide, when said target site is occupied by a ligand for said target polypeptide, which method comprises:

(a) providing a target polypeptide comprising a target site to which is bound a ligand to form a target complex, the ligand being bound to an adaptor moiety;

(b) providing a selected binding polypeptide which binds specifically to the adaptor moiety;

(c) modifying one or more regions of the selected binding polypeptide other than regions that interact with the adaptor moiety, to obtain a first plurality of candidate binding polypeptides;

(d) contacting the target complex with the first plurality of candidate binding polypeptides under conditions that are sub-optimal for binding of the unmodified selected binding polypeptide to the adaptor moiety;

(e) selecting a candidate binding polypeptide that binds simultaneously to the moiety molecule and the target polypeptide;

(f) modifying at least a part of a region of the selected candidate binding polypeptide that interacts with the adaptor moiety to obtain a second plurality of candidate binding polypeptides;

(g) contacting the second plurality of candidate binding polypeptides with the target polypeptide bound to the ligand in the absence of the adaptor moiety; and

(h) selecting a candidate binding polypeptide which binds to the target polypeptide.

18. A method for selecting an antibody that binds to a target antigen only when the antigen is present in an antibody-antigen complex, which method comprises:

(a) providing a first antibody which binds specifically to a hapten;

(b) providing a second antibody which binds specifically to the target antigen;

(c) modifying one or more regions of the paratope of said first antibody which do not contact the hapten when the unmodified antibody is bound to the hapten, to obtain a first plurality of modified antibodies;

(d) contacting the first plurality of modified antibodies with an antibody-antigen-hapten complex comprising the second antibody, the hapten and the target antigen under conditions that are sub-optimal for binding of the unmodified first antibody to the hapten;

(e) selecting a candidate antibody that binds simultaneously to the hapten and the second antibody;

(f) modifying at least a part of a region of the selected candidate antibody that interacts with the hapten to obtain a second plurality of candidate antibodies;

(g) contacting the second plurality of candidate antibodies with the second antibody bound to the target antigen in the absence of the hapten; and

(h) selecting a candidate binding molecule which binds to the second antibody.

Application No:	GB 0309397.8	**Examiner:**	Dr Rowena Dinham
Claims searched:	All	**Date of search:**	22 October 2003

Patents Act 1977 : Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
A		WO 02/068691 A1 (ISIS PHARMACEUTICALS) See entire document, especially page 9 line 7-20 and Examples
A		*Nucl Acids Res*; Vol 31, pp e23 (2003). Tanaka *et al.* "De novo *production of diverse intracellular antibody libraries*" See entire document
A		US 4778751 (EL SHAMI) See entire document, especially column 3 line 48-20

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art
Y	Document indicating lack of inventive step if combined with one or more other documents of same category	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

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Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[V]:

Worldwide search of patent documents classified in the following areas of the IPC[7]:

C07K; G01N

The following online and other databases have been used in the preparation of this search report :

WPI, EPODOC, JAPIO, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS